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ORIGINAL ARTICLE

Hybrid incompatibilities in the parasitic wasp genus *Nasonia*: negative effects of hemizygosity and the identification of transmission ratio distortion loci

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The occurrence of hybrid incompatibilities forms an important stage during the evolution of reproductive isolation. In early stages of speciation, males and females often respond differently to hybridization. Haldane's rule states that the heterogametic sex suffers more from hybridization than the homogametic sex. Although haplodiploid reproduction (haploid males, diploid females) does not involve sex chromosomes, sex-specific incompatibilities are predicted to be prevalent in haplodiploid species. Here, we evaluate the effect of sex/ploidy level on hybrid incompatibilities and locate genomic regions that cause increased mortality rates in hybrid males of the haplodiploid wasps *Nasonia vitripennis* and *Nasonia longicornis*. Our data show that diploid F_1 hybrid females suffer less from hybridization than haploid F_2 hybrid males. The latter not only suffer from an increased mortality rate, but also from behavioural and spermatogenic sterility. Genetic mapping in recombinant F_2 male hybrids revealed that the observed hybrid mortality is most likely due to a disruption of cytonuclear interactions. As these sex-specific hybrid incompatibilities follow predictions based on Haldane's rule, our data accentuate the need to broaden the view of Haldane's rule to include species with haplodiploid sex determination, consistent with Haldane's original definition.
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Keywords: hybrid incompatibilities; Haldane's rule; haplodiploidy; speciation; cytonuclear interaction; transmission ratio distorting loci

INTRODUCTION

The identification of hybrid incompatibilities, measured as increased sterility and mortality of hybrids, is a major goal in evolutionary biology as they cause strong selection pressure against the formation of hybrid offspring and thus are a potential driving force of speciation. Hybrid incompatibilities are caused by disrupted epistatic gene interactions in hybrids, also known as Dobzhansky–Muller (DM) interactions (Turelli and Orr, 1995), and in a few cases (mainly in *Drosophila*), hybrid incompatibility genes have been identified (reviewed by Presgraves, 2010). Although no general pattern in pathways and genes that are particularly prone to hybrid incompatibility has yet been discerned, it has long been known that 'when in the offspring of two different animal races one sex is absent, rare or sterile, that sex is the heterozygous (heterogametic, that is, XY or ZW) sex' (Haldane, 1922). This pattern, referred to as Haldane's rule, is often observed in hybrids of recently diverged populations or species (Coyne and Orr, 1997). There are three main genetic theories that explain why especially the heterogametic sex should suffer from hybridization (Orr, 1997): The faster-X theory, the faster-male theory and the dominance theory. All three theories assume that, owing to their shared evolutionary history, epistatic genes are co-adapted within species but not between species. Thus, upon hybridization, these co-adapted genes are replaced by gene variants that have never been selected to interact properly with each other, and their interactions in hybrids are likely disrupted (DM interactions).

The faster-X theory assumes that beneficial recessive mutations accumulate more easily under haploidy. If true, genes on the sex chromosomes of diploid organisms should on average evolve faster than genes on the autosomes, because the sex chromosomes go through rounds of haploidy in the heterogametic sex (Charlesworth *et al.*, 1987). Epistatic interactions of genes on a fast-evolving chromosome are more disruption-prone when brought into a foreign genetic background than genes on slowly evolving autosomes, because of their larger divergence. Therefore, the heterogametic sex is more likely to suffer from hybridization than the homogametic sex, because the latter can be saved by heterozygosity of the less compatible loci. Research has shown the complexity of sex chromosome evolution, as both supporting (for example, Musters *et al.*, 2006; Baines *et al.*, 2008) and opposing (for example, Betancourt *et al.*, 2002; Thornton *et al.*, 2006; Mank *et al.*, 2010) evidence for faster evolution of the X-chromosome has been found (reviewed for *Drosophila* by Presgraves, 2008; Singh *et al.*, 2009).

The faster-male theory states that male traits evolve faster than female traits owing to stronger sexual selection on males. This leads to Haldane's rule under male heterogamety, because more diverged male genomes have a higher chance to suffer from DM interactions than less diverged female genomes (Wu and Davis, 1993). Several studies have found support for the faster-male theory (Civetta and Singh, 1995; Meiklejohn *et al.*, 2003; Zhang *et al.*, 2004; Eads *et al.*, 2007;

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Malone and Michalak, 2008). A second cause underlying the faster-male theory is that spermatogenesis is a sensitive process, easily disrupted by mutations that lead to male sterility, whereas mutations have less effect on oogenesis and hence female sterility (Wu and Davis, 1993). This leads to more cases of hybrid male sterility than female sterility (reviewed by Schilthuizen *et al.*, 2011). One major problem of the faster-male theory is that it can only explain Haldane's rule under male heterogamety. Under female heterogamety other factors (such as dominance, see below) have to be invoked that overcome 'faster-male' effects (Wu and Davis, 1993).

The dominance theory assumes that mutations are (partially) recessive and thus masked by heterozygosity (Turelli and Orr, 1995). In hybrids, gene interactions can be disrupted throughout the whole genome when diverged genes are forced to interact, but DM interactions are often rescued by dominance effects of the autosomes under diploidy. However, when the interactions involve sex-linked genes, the heterogametic sex is not saved by heterozygosity of the sex chromosome and thus has a higher chance to suffer from hybrid incompatibilities than the homogametic sex. This theory explains Haldane's rule for both male and female heterogamety, and is supported by several studies that have found a major effect of dominance in hybridizations that follow Haldane's rule (for example, True *et al.*, 1996; Jiggins *et al.*, 2001; Presgraves, 2003; Tao and Hartl, 2003; Bierne *et al.*, 2006).

The general view is that Haldane's rule should be considered to be a composite phenomenon with multiple underlying mechanisms (Wu *et al.*, 1996). What underlines this view is that some studies have found multiple mechanisms explaining their results on hybrid incompatibilities and Haldane's rule (for example, Hollocher and Wu, 1996). Although additional mechanisms have been proposed to explain Haldane's rule, the faster-male and the dominance theory are most supported (reviewed by Kulathinal and Singh, 2008). However, if hybrid incompatibilities are caused by recessive DM interactions, studying them can become a challenge because most incompatibilities are masked in diploid hybrid offspring. As a consequence, laborious introgression studies are necessary to investigate these recessive incompatibilities in diploid model organisms (Hollocher and Wu, 1996; True *et al.*, 1996; Tao and Hartl, 2003; Tao *et al.*, 2003; Masly and Presgraves, 2007).

Haplodiploid species, where fertilized and unfertilized eggs develop into females and males, respectively, are promising genetic models for studying hybrid incompatibilities when dominance effects are expected. Males express only one allelic variant, either dominant or recessive, and are thus very useful for the identification of negative epistatic gene interactions that cause hybrid incompatibilities on a genome-wide scale (Gadau *et al.*, 1999; Ellison *et al.*, 2008; Niehuis *et al.*, 2008). Haldane himself referred to haplodiploids as a group in which the pattern that he had described, that is, Haldane's rule, should be prevalent. He stated: 'groups in which the male sex is haploid are only extreme cases of the normal type, in that all the chromosomes here behave like sex chromosomes of other groups' (Haldane, 1922, page 101). Although recognized as a group that could be instrumental in unravelling the mechanisms that underlie Haldane's rule, haplodiploids have so far been deemed not to 'fall under the Haldane's rule banner' because of their lack of heteromorphic sex chromosomes (Kulathinal and Singh, 2008). In line with the view of Haldane himself, Koevoets and Beukeboom (2009) argued that Haldane's observation of the differential effect of hybrid incompatibilities in males versus females should be expanded to include species with haplodiploid sex determination for two reasons: first, the inheritance of the complete haplodiploid genome is comparable to the inheritance of sex chromosomes in diploids; and second, all three mechanisms that explain Haldane's rule apply under haplodiploidy in that males are predicted to suffer more than females (that is, males would suffer under the dominance theory because of haploidy; under the faster-male theory because of faster evolution by sexual selection and under the faster-X theory because of faster evolution by natural selection). Moreover, having more genes (or chromosomes) that inherit in a haplodiploid manner (such as sex chromosomes) would lead to Haldane's rule faster. This is illustrated in *Drosophila* where sister species with larger X-chromosomes suffer from Haldane's rule faster than sister species with smaller X-chromosomes (Turelli and Begun, 1997).

Until now, only a single haplodiploid species pair (that is, *Nasonia vitripennis* and *Nasonia giraulti*) has been screened for sex-specific hybrid incompatibilities (Breeuwer and Werren, 1995). The authors found no incompatibilities in F_1 hybrid females (although not systematically tested), but large mortality and slight sterility in F_2 hybrid males. However, a problem when studying Haldane's rule in haplodiploids is that male and female hybrids are not formed in the same generation and thus cannot be tested under the same ploidy level. As in diploids, hybrid females in haplodiploids form in the F_1 generation. Hybrid males, however, arise as offspring of hybrid females. Although we refer to these hybrids as F_2 males, they are actually the first-generation male hybrids, and this is the generation that needs to be compared to F_1 hybrid females when testing for the occurrence of hybrid incompatibilities (Koevoets and Beukeboom, 2009). Creating backcross F_2 females is not a more appropriate comparison with F_2 males, because it involves comparing hemizygous males to partially heterozygous females and confounds the comparison of males and females with regard to dominance effects.

The parasitoid wasp genus *Nasonia* has proven a valuable model system for studying complex genetic traits owing to its haplodiploid sex determination system and available genome sequences (Werren *et al.*, 2010). The genus consists of four sister species, *Nasonia vitripennis*, *Nasonia longicornis*, *Nasonia giraulti* and *Nasonia oneida* (Darling and Werren, 1990; Raychoudhury *et al.*, 2010a). All *Nasonia* species are reproductively isolated from each other owing to infections with different *Wolbachia* strains, which can be cured with antibiotics. Breeuwer and Werren (1995) found that F_1 hybrid females from a cross between *Wolbachia*-cured *N. vitripennis* and *N. giraulti* are viable and fertile. Hybrid males had higher mortality rates than pure strain males, in particular hybrids with *N. giraulti* cytoplasm. The mortality occurred predominantly during larval development. The authors also found that surviving hybrids with *N. vitripennis* cytoplasm were mostly fertile, but those with *N. giraulti* cytoplasm were largely sterile. The asymmetry in the mortality and sterility rates of the hybrids was the first evidence for incompatibility between nuclear and cytoplasmic factors as a major cause of hybrid incompatibility in *Nasonia*.

Niehuis *et al.* (2008) investigated the increased mortality in hybrids of *N. vitripennis* and *N. giraulti* by analysing marker transmission ratio distortion in F_2 hybrid males. Transmission ratio-distorting loci (TRDLs) deviate from the expected 1:1 transmission ratio of the parental alleles and can indicate differential mortality that relies on the genotype at the TRDL. The authors localized cytotype-dependent TRDLs on chromosomes 1, 2, 4 and 5 (*Nasonia* has five chromosomes). Overall, more TRDLs were incompatible with *N. vitripennis* cytoplasm than with *N. giraulti* cytoplasm. This is in contrast to the mortality found by Breeuwer and Werren (1995). Given this asymmetry in incompatibility, Niehuis *et al.* (2008) concluded that interactions between nuclear and cytoplasmic genes cause the mortality of F_2 hybrid males. Clark *et al.* (2010) measured sterility in reciprocal F_2 male hybrids and found disrupted mating behaviour and fewer sperm

numbers than in pure species males, with *N. girault* cytoplasm leading to more sterility than *N. vitripennis* cytoplasm. By contrast, Bordenstein *et al.* (2001) found no increased mortality or sterility in hybrids between *N. girault* and *N. longicornis*. This can be explained by the short divergence time of these two sister species (0.2 mya; Campbell *et al.*, 1993). The availability of four *Nasonia* species, with different divergence times and different levels of pre- and post-zygotic isolation, makes *Nasonia* particularly useful for speciation research, because it allows tracing the evolution of hybrid incompatibilities in this genus.

Here we use hybrids of *N. vitripennis* and *N. longicornis* to for the first time systematically compare female F_1 and male F_2 mortality and sterility levels, and to document sex-specific differences in hybrid incompatibilities between these haplodiploid species. Furthermore, we study and map hybrid incompatibilities in the F_2 hybrid males and compare our data on mortality, sterility and the location of TRDLs with previous studies that used different interspecific crosses of *Nasonia* in order to infer the evolutionary history of the incompatibilities in this species complex.

MATERIALS AND METHODS

We used the *N. vitripennis* strain AsymC (origin: Leiden, The Netherlands) and the *N. longicornis* strain IV_7R_2 (origin: UT, USA) for the cross experiments. Both strains are cured from their *Wolbachia* infection and are highly inbred. Wasps were reared on *Calliphora vicina* hosts at 25 °C under constant light.

Mortality estimates

The experimental setup is summarized in Supplementary Figure S1. Virgin male (±24 h old) and female wasps (±72 h old and kept on hosts for feeding and to initiate egg-laying) were set up individually in four different crosses (♂×♀): *N. vitripennis*×*N. vitripennis*, *N. longicornis*×*N. longicornis*, *N. longicornis*×*N. vitripennis* and *N. vitripennis*×*N. longicornis*. These crosses are referred to as VV[V], LL[L], LV[V] and VL[L], respectively, with the first letter indicating the paternal genome complement, the second letter indicating the maternal genome complement, and the letters V and L with the square brackets indicating the cytotype. The mating pairs were left to mate for 24 h at 25 °C, after which the males were removed and the females provided with two hosts for 24 h at 25 °C (experimental day 1). The hosts were replaced every 24 h for 3 days, but only offspring from hosts of experimental days 2 and 3 were used for the experiment. Because there is high embryo mortality after egg counting owing to opening of the host puparium, the mating pairs were divided into two groups for each cross: one to count the number of oviposited eggs and one to count the number of eclosed adults. Hosts from the females in the egg-count group were submerged in Carnoy's fixative (ethanol:glacial acetic acid=1:3) and stored at −20 °C for at least 48 h. Hosts from the adult-count group were incubated at 25 °C and F_1 adult offspring was counted in the black pupal stages (2–3 days prior to their eclosion after opening the host puparium) and separated into males and females to obtain virgins. It is assumed that wasp pupae collected in this stage would later eclose as adults, because the hybrid mortality occurs in earlier life stages (Breeuwer and Werren, 1995). Eclosion of counted wasps was near to 100%, but not systematically recorded. F_1 virgin females were kept on fresh hosts upon eclosion for ±48 h for host feeding and to initiate egg-laying. After 48 h, three virgin females per mating pair were randomly assigned to one of three groups for (1) egg counting, (2) adult counting and (3) embryo collection (eggs) for DNA analysis. Hosts were replaced every 24 h for 3 days, from which only experimental days 2 and 3 were used for the experiment. After every 24 h, the hosts were removed and either submerged in Carnoy's fixative and stored at −20 °C for at least 48 h (group-1), replaced at 25 °C (group-2) or opened to remove the unhatched eggs (group-3). The F_2 adult offspring were counted and collected 2–3 days prior to their eclosion. For the analysis of F_1 and F_2 mortality, the average offspring production per female was determined over all pupae parasitized on experimental days 2 and 3. Diapause larvae, larvae that were very retarded in their development and dead individuals were removed from the data set (<12%).

Sterility estimates

The level of F_1 female sterility was determined by counting the proportion of F_1 virgin females that produced F_2 offspring. F_2 male sterility was determined both behaviourally and spermatogenically. After being removed from the host as late instar pupae, F_2 males were kept in plastic vials in groups of ~15 individuals until the sterility experiment that took place 24 h after eclosion. The male's mating behaviour was observed in isolation with a virgin *N. vitripennis* female, except for *N. longicornis* males, which were offered a virgin *N. longicornis* female. Previous experiments have shown that *N. vitripennis* virgin females are more appropriate for testing the mating behaviour of VV[V], LV[V] and VL[L] F_2 males because of the strong mating discrimination of *N. longicornis* virgin females, whereas LL[L] F_2 males are better tested with *N. longicornis* virgins (Koevoets, unpublished data). Virgin females were put in test vials at least 1 h prior to the sterility experiment. The male was introduced to the female and observed for 10 min at 25 °C. Different behavioural categories were scored (Supplementary Table S2). Females that mated with a male within the 10-min observation period were isolated for 24 h after which they were given three hosts to screen their progeny. The absence of females in their progeny indicates male spermatogenic sterility, although other post-mating–pre-zygotic isolation factors (such as the inability to transfer sperm) could also explain a lack of female progeny. Therefore, the measure of spermatogenic sterility also includes these other post-mating–pre-zygotic isolation factors. All unmated females were discarded, and all tested males stored at −20 °C until DNA extraction.

Genotyping

F_2 hybrid male embryos were collected ⩽24 h after oviposition by a virgin F_1 female. The host puparium was removed and the embryos were transferred individually to 80-μl digestion buffer (Maniatis *et al.*, 1982) (without proteinase-K) and ground with a sterile needle. DNA extraction was initiated by adding 20 μl of digestion buffer containing 40 μg of proteinase-K to the samples. Subsequent steps followed the standard high salt-chloroform protocol (Maniatis *et al.*, 1982). The DNA was dissolved in 20 μl of MilliQ water. The DNA of the F_2 hybrid adult males was extracted by following the regular high salt-chloroform protocol and the DNA was dissolved in 20 μl of MilliQ. Microsatellite markers were amplified by using the Qiagen multiplex PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations (PCR profile: 15 min at 95 °C, followed by 30 cycles of 30 s at 94 °C, 1.5 min at T_A and 1 min at 72 °C, followed by 45 min at 72 °C). We used eight multiplex sets of 6 or 7 microsatellite markers each (Supplementary Table S3). All stock DNA was diluted 10×, from which 2 μl was used for embryonic DNA PCR and 1 μl for adult DNA PCR. All reactions were performed in 5-μl volumes using Applied Biosystems Veriti or Applied Biosystems 9700 thermocyclers. Fragments from embryonic PCR were diluted 20 times and from adult PCR 400 times, separated on the Applied Biosystems 3730 DNA Analyzer and analysed using GeneMapper v4.0 (Applied Biosystems, Carlsbad, CA, USA).

Statistical analyses

Linkage mapping. The linkage map was inferred by using the segregation data of 201 F_2 hybrid embryos from both reciprocal hybrid crosses, using JoinMap (version 3.0; van Ooijen and Voorrips, 2001). Markers were grouped using a minimum logarithm of odds score of 7 and a maximum recombination fraction of 0.450. Markers with insufficient linkage were removed from the analysis. Following Niehuis *et al.* (2008), Haldane's mapping function was used to translate recombination fractions into map distances in centimorgans (cM).

Segregation bias. The segregation bias for all markers was tested by χ^2 goodness of fit tests (df=1) against the expected segregation of 1:1 of parental alleles. Both Yates and Bonferroni corrections were applied. Furthermore, we used a Bayesian multipoint mapping approach as implemented in the software ANITA (Vogl and Xu, 2000). Interactions between nuclear markers were tested by χ^2 goodness of fit tests (df=1), which detect deviations between the observed and the expected genotypes based on the allele frequencies in the two adult hybrid data sets. We refrained from testing makers that map to the same chromosome because of linkage. To assess significance, we simulated 10 000 random populations of 125 hybrid individuals each and with a marker distribution as in our experiments with the aid of a Perl script. For each population, we recorded the highest χ^2-value

and generated a frequency histogram. We then inferred the χ^2 threshold value that was exceeded in less than 5% of the random populations (that is, 12.304).

Mortality. The mortality level was determined by an indirect egg-to-adult measurement by comparing the number of eggs produced in one group to the number of adults produced in another group, by using the Mann–Whitney *U*-test (MWU test) in SPSS 14.0. The survival probability (*Z*) was estimated from the ratio of the number of adults (Y) divided by the number of eggs (X). The variance in the survival probability was calculated from the variances of the number of eggs and adults according to Breeuwer and Werren (1995) using the formula

$$VAR(Z) = \frac{VAR(Y)}{\mu_X^2} + VAR(X)\frac{\mu_Y^2}{\mu_X^4}$$

The properties of the normal distribution were used to determine confidence intervals for the different survival probabilities at α=0.05.

Sterility. The mating behaviour of males was scored, classified into seven successive categories and analysed following Clark *et al.* (2010). The transition probability was calculated as the frequency of males that perform a specific behaviour in category *a* that also perform the behaviour in category *a+1*. Deviations between crosses in the transition from one category to the next were determined by 2×4 χ^2 tests with Bonferroni correction. When a significant difference was found, all the transitions were tested per cross in a pairwise manner, using 2×2 χ^2 goodness of fit or Fisher's exact tests, depending on whether χ^2 assumptions were violated (fewer than 50 samples, or expected values below 5). To compare the percentage sterility of the different crosses (for behavioural, spermatogenic and total sterility), we performed χ^2-tests on proportions to see if the level of sterility depends on the cross type. When true, a Tukey-type multiple comparison was performed to test which crosses were significantly different (Zar, 1999). It was also determined whether there is a tendency for hybrids that carry more *N. vitripennis* alleles on each of the five chromosomes to be more or less often sterile. For this, the genotypes of fertile and (spermatogenically) sterile hybrids were compared. The data for all markers were merged per chromosome and the number of V alleles that was found per chromosome was tested for sterile versus fertile males of both hybrid types by using a 2×5 χ^2 goodness of fit. All tests were performed by using Microsoft Excel 2003 and GraphPad online (GraphPad Software, La Jolla, CA, USA).

RESULTS

Linkage map construction

We inferred a linkage map based on the marker segregation data from 201 F_2 hybrid male embryos after discarding individuals for which more than 20% of the markers failed to amplify. Data from the reciprocal hybrid crosses were pooled because we did not observe marker transmission ratio distortion at the embryonic stage in either mapping population (see below). We deliberately left two additional markers (that is, Nv324 and Nv344) in the final data set despite the fact that they did not map reliably, as we knew their position on the linkage map (chromosome-5 near marker Nv125). Five markers (out of the initial 55) were removed from the data set because they failed to amplify in more than 20% of the embryonic samples. The final linkage map consisted of 48 microsatellite markers (Supplementary Table S3), spread over five linkage groups (Figure 1), that were assigned to the five chromosomes of *Nasonia* using four anchored microsatellites (according to Rütten *et al.*, 2004), The total map length was 490 cM and approximates the full *Nasonia* genome of 295 Mb (Werren *et al.*, 2010).

F_1 mortality

We found significant differences between the number of eggs laid and the number of emerging adults in all analysed crosses, except the

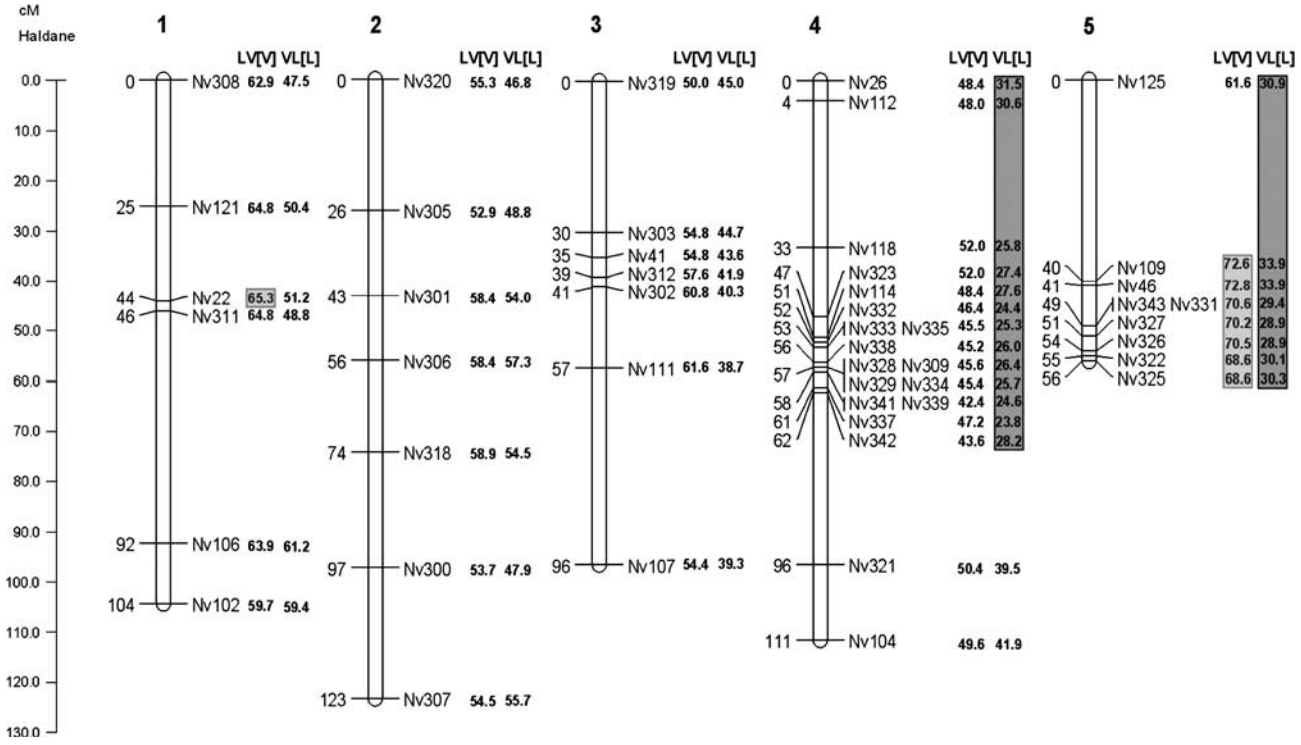

Figure 1 *N. vitripennis* and *N. longicornis* linkage map with allelic recovery rates. The linkage map is based on the microsatellite marker segregation in F_2 hybrid embryos from pooled reciprocal hybrid crosses (*n*=201). Recombination distances are shown in Haldane centimorgans on the left and markers on the right of each chromosome. The recovery rates of the *N. vitripennis* and *N. longicornis* alleles in the F_2 adult hybrids are shown next to the chromosomes for both crosses and are expressed as the percentage of V alleles. The markers with a significant segregation distortion are indicated in light grey (for LV[V]) and dark grey (for VL[L]) shaded boxes. Chromosome numbers are according to Rütten *et al.* (2004).

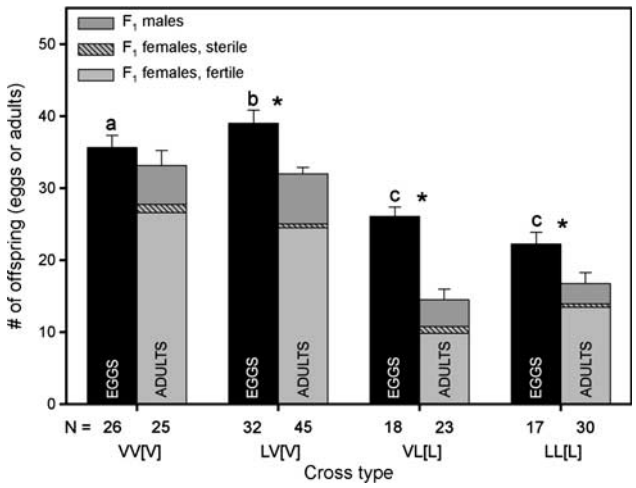

Figure 2 F_1 mortality. F_1 mortality in the hybrid and pure species crosses. Cross type on the *x*-axis represents the parental cross. Mortality is measured as indirect egg-to-adult survival probability. The black bars represent the number of eggs and the grey bars represent the number of adults (dark grey for male and light grey for female offspring). Female offspring is distinguished in sterile (patterned) and fertile (solid) females based on whether or not F_1 females produced F_2 males. Cross type represents ♂ × ♀[cytoplasm]. Sample sizes are indicated in the bars. * indicates significant difference between the number of eggs and adults per cross based on MWU test with α=0.05. The letters indicate different groups with regard to the number of eggs produced in the different crosses (MWU test, α=0.05).

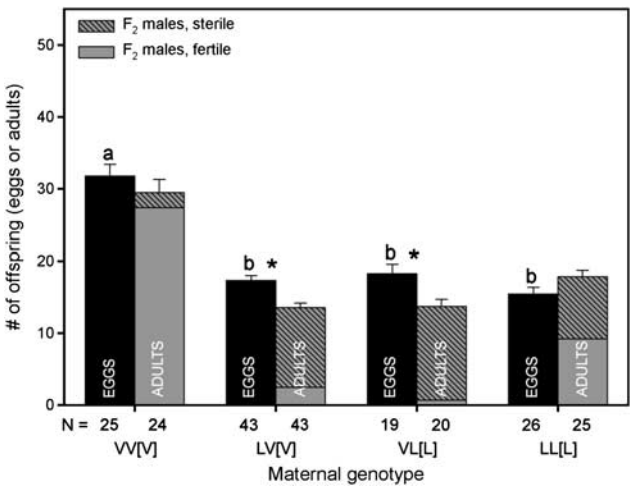

Figure 3 F_2 mortality. F_2 mortality in the hybrid and pure species crosses. Cross type on the *x*-axis represents the grand parental cross. Mortality is measured as indirect egg-to-adult survival probability. The black bars represent the number of eggs and the grey bars represent the number of male offspring. Male offspring is distinguished in sterile (patterned) and fertile (solid) males based on the sterility experiment. Cross type represents ♂ × ♀[cytoplasm]. Sample sizes are indicated in the bars. * indicates significant difference between the number of eggs and adults per cross based on MWU test with α=0.05. The letters indicate different groups with regard to the number of eggs produced in the different crosses (MWU test, α=0.05).

Table 1 95% confidence intervals of the survival probabilities for the hybrid and pure species crosses

Cross	*95% confidence interval*			
	F_1 (female-biased)		*F_2 (male)*	
VV[V]	85.8	99.6	85.5	99.9
LV[V]	76.9	87.2	72.2	84.2
VL[L]	49.8	61.3	68.8	81.9
LL[L]	66.9	83.9	106.3	124.2

Survival is calculated over the total offspring count: in the F_1 these are male and female (sex ratio greatly biased towards female), and in the F_2 these are only male.

N. vitripennis pure strain control (VV[V]; Figure 2). As all strains that were used in our experiment are genetically homogeneous, all F_1 female offspring from a given type of cross has the same genotype. This means that genetic variance contributes little to the phenotypic differences of the F_1 hybrids and that the observed variance in their survival is largely environmental. This is also underlined by the significant mortality in the pure *N. longicornis* cross LL[L], which disappears in the next generation that was cultured on a different batch of hosts (see below). Because of haplodiploidy, F_1 males from the VV[V] and LV[V] crosses are pure strain *N. vitripennis*, and F_1 males produced in the LL[L] and VL[L] crosses are pure strain *N. longicornis*. The number of pure species males did not differ between crosses (MWU test VV[V] versus LV[V], P=0.269; LL[L] versus VL[L], P=0.971), which shows that F_1 mortality is due to a reduction of F_1 females (how this reconciles with the presence of sex-specific mortality will be discussed later). Table 1 shows the 95% confidence intervals for the F_1 survival probabilities of the hybrid and pure species crosses. Based on non-overlapping confidence intervals, only F_1 offspring from the VL[L] cross have lower survival than offspring from the other three crosses, the survival of the LV[V] cross did not differ significantly from either of the pure species.

F_2 mortality

F_2 hybrid males suffered from significant mortality (Figure 3). As the genome of F_2 hybrid males is a product of recombination between the two chromosomal sets of the mother, each hybrid male has a unique nuclear genome. Figure 3 shows that F_2 hybrid male mortality is slightly higher in the VL[L] hybrids than in the LV[V] hybrids, with the only genetic difference between them being their cytoplasm. Table 1 shows the 95% confidence intervals for the F_2 survival probabilities of the hybrid and pure species crosses. The pure species F_2 males have very high survival (in *N. longicornis* even over 100% owing to independent samples within and between groups, but the number of adults is not significantly higher than the number of eggs), whereas the hybrid F_2 males have a lowered survival probability that is similar for both reciprocal hybrid crosses.

F_1 female sterility

Female sterility was estimated as the proportion of F_1 females that did not produce F_2 males. This proportion was used to infer the ratio of fertile and sterile F_1 females in our data set (Figure 2). F_1 female sterility did not differ significantly across all four crosses ($P>0.05$ χ^2 on proportions (Zar, 1999)). The egg production of hybrid females was similar to pure *N. longicornis* females, but reduced compared with pure *N. vitripennis* females (Tukey test, $P<0.05$; see letters in Figure 3 for differences in F_2 egg production by F_1 females).

F_2 male sterility

We tested a subset of F_2 males for behavioural sterility by recording their courtship behaviour for seven different behavioural categories

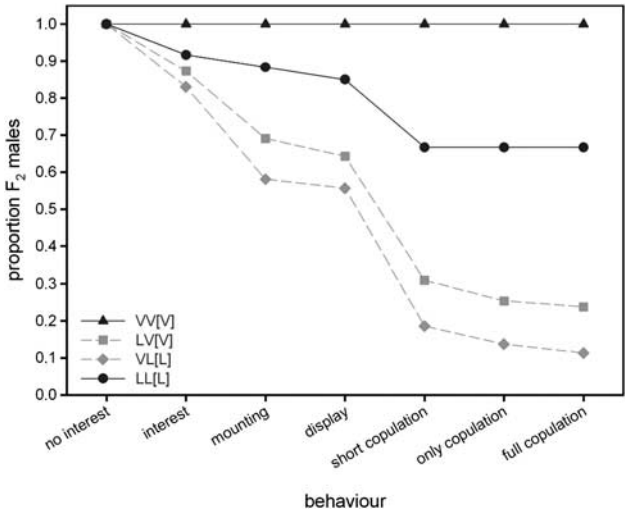

Figure 4 F_2 male behavioural sterility. Behavioural sterility of F_2 males from hybrid and pure species crosses measured as the ability of males to show different categories of the male mating behaviour. The cumulative proportion of males that showed a particular behaviour is plotted against the behavioural category.

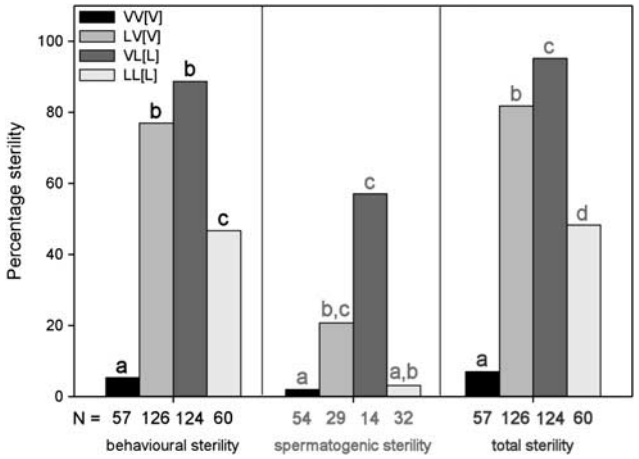

Figure 5 F_2 male sterility. F_2 male sterility for three different types of sterility: behavioural sterility, spermatogenic sterility and total sterility (combining different sterilizing factors). The percentage of spermatogenic sterility is based on mated males only, because for the unmated males the spermatogenic sterility could not be determined. Sample sizes are indicated in the bars; the letters indicate a significant difference between the different types of males for a particular type of sterility based on a Tukey test for multiple comparisons (α=0.05) (different shadings of letters indicates which values have been tested against each other).

Table 2 Transition probabilities between different categories of male courtship behaviour in the hybrids and pure species

F_2 ♂♂	n	*Interest*	*Mounting*	*Display*	*Short cop.*	*Only cop.*	*Full copulation*
VV	57	1.00[a]	1.00[a]	1.00	1.00[a]	1.00[a]	1.00[a]
LV	126	0.87[b]	0.79[b]	0.93	0.48[b]	0.82[b]	0.94[a.b]
VL	124	0.83[b]	0.70[b]	0.96	0.33[b]	0.74[b]	0.82[b]
LL	60	0.92[a.b]	0.96[a]	0.96	0.78[c]	1.00[a]	1.00[a]
	P	**0.008**	**<0.0001**	0.241	**<0.0001**	**<0.0001**	**0.002**

The bold *P*-values indicate a significant difference (α=0.05; χ^2 or Fisher's exact test) between the probabilities within a category. The letters indicate within a category which lines differ from each other in the transition from one category to the next; that is, the letters indicate per behavioural category which lines have the same courtship behaviour.

(Supplementary Table S2) in isolation with a single virgin female for 10 min. The results show that nearly all pure strain males showed normal courtship, whereas many hybrid males showed aberrant courtship behaviour (Figure 4). Table 2 shows the probabilities of males performing a typical sequence of courtship behaviours, ranging from showing interest in the female to performing a copulation with post-copulatory behaviour. When males show normal courtship, they transit from the first to subsequent categories with high probabilities. Hybrid males showed significantly lower transition rates for all categories, except from 'mounting' to 'display'. This means that hybrid males that were able to mount a female were also able to display courtship to that female. In almost all cases, the two pure crosses showed similar transition probabilities to each other, as did the two hybrid crosses. When combining all the aberrant courtship behaviours that did not lead to a successful copulation, we get a measure of behavioural sterility (Figure 5). The graph shows that hybrid males tend to be significantly more often sterile than males of the two pure species (Tukey-type multiple comparison, α=0.05)

We estimated the level of spermatogenic sterility of F_2 males by scoring the daughter production of those males that had successfully copulated (Figure 5). The estimated level of spermatogenic sterility in pure species males is less than 3%. The level of spermatogenic sterility in hybrid males is much higher, with the highest level for VL[L] males (57% compared with 21% for LV[V]). Using a Tukey-type multiple comparison to determine which crosses differed from each other, we found that spermatogenic sterility (Figure 5) of the two hybrid crosses is higher than that of the pure VV[V] cross, but the level of sterility in the LV[V] cross is equal to pure LL[L].

Combining behavioural and spermatogenic sterility gives a measure for total sterility of F_2 males (Figure 5). A large percentage of hybrid F_2 males are sterile, whereas the percentage of sterile F_2 pure males is much smaller. All types of males differed significantly from each other in the percentage of total sterility (Tukey-type multiple comparison, α=0.05). Hybrids with *N. longicornis* cytoplasm had a higher level of sterility than hybrids with *N. vitripennis* cytoplasm. This is also reflected by the genotypic data (described in more detail below). When comparing the genotypes of fertile and sterile males from the two hybrid crosses for the occurrence of *N. vitripennis* alleles on the five different chromosomes, only the VL[L] data set shows a strong bias towards *N. longicornis* alleles in fertile males ($P<0.0001$). This effect is evident for loci on chromosomes 2–5, but strongest for chromosomes 2 and 5.

Transmission ratio-distorting loci

After the F_2 hybrid males were tested for sterility, they were genotyped with the 50 microsatellite markers that were used to construct the linkage map (markers Nv324 and Nv344 were also used, although they could not be mapped). A total of 126 LV[V] and 124 VL[L] F_2 males were analysed, from which only one LV[V] sample was removed because of bad amplification. Each marker was tested for a deviation from the expected ratio of 1:1 of parental alleles by using χ^2-tests after Yates and Bonferroni corrections.

The marker segregation data of the LV[V] hybrids suggested a TRDL on chromosome-1 at marker Nv22 and at all markers on chromosome-5 in the region between and including Nv109 and Nv325. In all cases the distortion was in favour of the *N. vitripennis* (V) allele. No significant marker segregation bias was found on chromosomes 2, 3 and 4. Marker segregation data in hybrids from

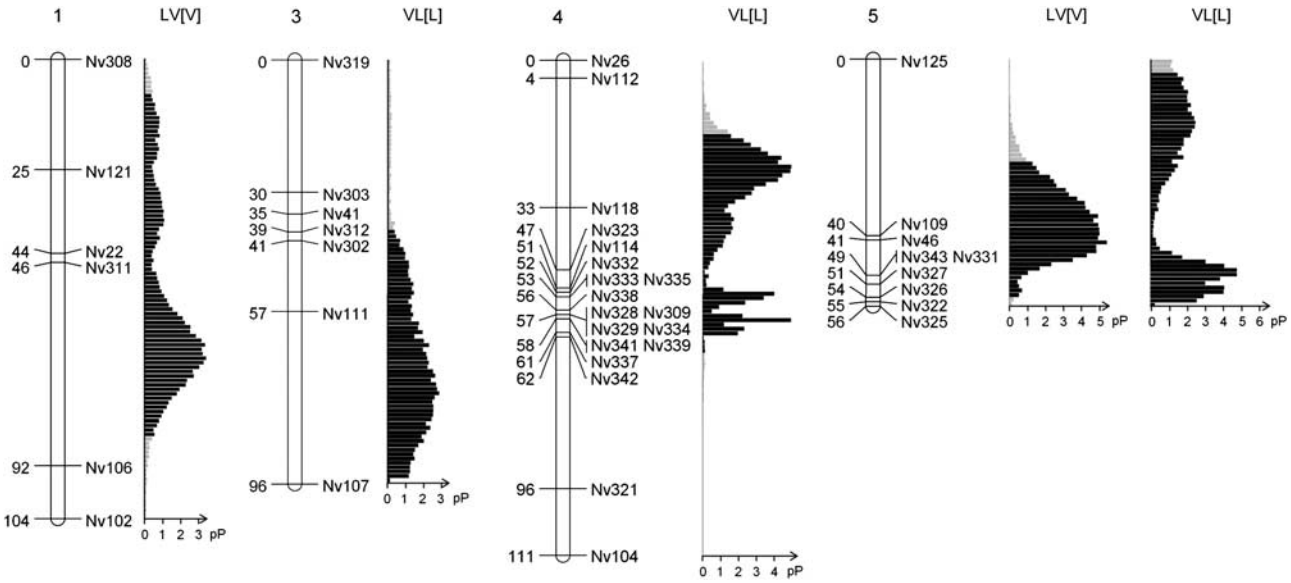

Figure 6 Estimated position of predicted TRDLs in hybrid F_2 males. Shown are density distributions of the pP for the position of the TRDLs in adult male F_2 hybrids (chromosome-2 is not shown because it lacks TRDLs). Each distribution was calculated from 20 000 Markov chain Monte Carlo samples taken from the stationary phase and assuming a Poisson prior (λ=0.5) for the number of TRDL. The pP values for these distributions of single TRDLs were 73.00, 62.79, 47.46, 75.20 and 59.73%. The hybrid cross in which the TRDL distribution was found is indicated above the distribution. Chromosome numbers are according to Rütten *et al.* (2004).

the reciprocal cross (VL[L]) suggested a TRDL on chromosome-4 from and including marker Nv26 to Nv342 and on chromosome-5 for all the markers. In all cases the distortion was in favour of the *N. longicornis* (L) allele. Chromosomes 1, 2 and 3 were not distorted in their marker segregation. The distortion is illustrated in Figure 1 as the percentage of V alleles in the F_2 males of each hybrid cross. It is evident that in both hybrid crosses the microsatellite alleles are distorted towards the species that delivered the cytoplasm (including the mitochondria): *N. vitripennis* for LV[V] and *N. longicornis* for VL[L].

To estimate the number of TRDLs that could explain the observed distortion in the genotypic data, we applied a Bayesian mapping approach. For the F_2 hybrids with *N. vitripennis* cytoplasm, it predicted a single TRDL on chromosome-1 with a posterior probability (pP) of 73% and a single TRDL on chromosome-5 with a pP of 75.2%. For the F_2 hybrids with *N. longicornis* cytoplasm, the Bayesian analysis was much less distinct. The analysis suggested one or two TRDLs on chromosome-4 with a pP of 47.5% and 43.2% respectively, and an additional TRDL on chromosome-5 (pP of 59.4%). The analysis for chromosome-3 revealed the presence of one TRDL based on the Bayesian method (pP of 62.8%), whereas the χ^2 failed to identify marker segregation distortion on that chromosome. The results of the Bayesian analysis with the number and location of the TRDLs are shown in Figure 6.

We tested whether the genotype of one TRDL was dependent on the genotype of another TRDL by applying a χ^2-test to the most distorted markers in our two F_2 hybrid data sets. In F_2 hybrids with *N. vitripennis* cytoplasm, we tested Nv22 (chromosome-1) and Nv46 (chromosome-4), and in F_2 hybrids with *N. longicornis* cytoplasm, we tested Nv337 (chromosome-4) and Nv326 (chromosome-5). The comparison of the observed recombinant and non-recombinant genotypes to those expected when the two respective markers segregate independently, revealed in neither case any evidence for a two-way interaction (P=0.20 and 0.48 for *N. vitripennis* and *N. longicornis* cytoplasm, respectively). Given that the Bayesian mapping approach of TRDLs suggested slightly different positions of the TRDLs for hybrids with *N. longicornis* cytoplasm (on chromosomes 4 and 5, and an additional TRDL on chromosome-3) compared with the χ^2 method, we conducted a $2\times2\times2$ χ^2-test on the genotypes of the markers Nv300, Nv46 and Nv302. Again, we found no evidence for an interdependence of the markers (P=0.16).

To assess the impact of nuclear–nuclear incompatibilities on hybrid mortality in general, we tested for significant deviations from the observed and expected ratio of recombinant and non-recombinant genotypes of all marker pairs for which the individual markers are on different chromosomes with the aid of a χ^2-test. To account for linkage, and thus interdependence of markers on a given chromosome and multiple testing, we simulated 10 000 random mapping populations with exactly the same marker order and marker distances as in our experiments, but with no marker segregation bias. Analysing the simulated data sets, we found χ^2-values larger than 12.304 for the conducted pairwise tests to occur in less than 5% of the cases. We therefore chose this value to assess significance in our F_2 hybrid populations. None of the χ^2-values from our F_2 hybrid genotypes with *N. longicornis* cytoplasm exceeded the threshold value of 12.304. However, the χ^2-tests of marker pairs in hybrids with *N. vitripennis* cytoplasm revealed a significant deviation from the expected values for marker pair Nv306 (chromosome-2) and Nv339 (chromosome-4) (χ^2=12.778).

DISCUSSION

We tested for the presence of sex-specific hybrid incompatibilities in the wasp genus *Nasonia* by investigating reciprocal crosses of *N. vitripennis* (V) and *N. longicornis* (L) to estimate mortality and sterility in males and females of pure strain and hybrid offspring. Compared with pure *N. vitripennis*, the F_1 female offspring of the hybrid crosses and the pure *N. longicornis* cross suffered from significant mortality. This mortality is, however, due to environmental factors rather than a disruption of gene interactions in hybrid females.

There are several arguments for this. First, mortality of the pure *N. longicornis* strain varied between generations and was not severe in the F_2 control cross. This is most likely due to variable host quality. Second, as all F_1 female offspring of a particular cross were genetically identical, any difference in mortality between individuals must have been environmentally induced. These observations are consistent with the biology of the species; *N. vitripennis* is a generalist parasitoid wasp and *N. longicornis* is a specialist of *Protocalliphora* blowfly pupae, a host species that we are unable to culture in the lab. *N. longicornis* is therefore more likely to be sensitive to fluctuations in host quality. In addition, the number of offspring produced by F_1 hybrid females was lower than that of pure strain *N. vitripennis*, but it was equal to that of pure strain *N. longicornis*. A likely reason for this is that *N. vitripennis* females have eight ovarioles, whereas *N. longicornis* females and the reciprocal hybrids have only six each (E Geuverink, personal communication).

Our data revealed that first-generation hybrid male (F_2) mortality was significantly larger than F_2 pure male mortality, but much smaller than that reported for hybrids of *N. vitripennis* and *N. giraulti* (Breeuwer and Werren, 1995). This difference in hybrid mortality between species pairs could be due to the different *Nasonia* species that were used, but also due to differences in experimental conditions (such as host species), as environmental factors appear to also affect the level of F_1 female mortality. This large effect of environmental conditions on the level of mortality obstructs the direct comparison of F_1 and F_2 mortality, because the subsequent generations experience different conditions. This is why in our experiment we always need to compare the hybrid mortality to the pure species mortality in the same generation in order to infer the level of mortality of the hybrids.

We found that F_2 male sterility was higher in hybrids than in pure strain individuals, whereas F_1 female sterility was not. The sterility test for pure LL[L] males differed from that of the other types of males in that *N. longicornis* rather than *N. vitripennis* virgin females were used. Nevertheless, the transition probabilities of the pure F_2 males were in most cases similar, which shows that both pure strain transition probabilities are a good control for the two hybrids. The F_2 hybrid male transition probabilities showed that hybrid males suffered greatly from behavioural sterility, similar to hybrids of *N. vitripennis* and *N. giraulti* (Clark *et al.*, 2010). As the transition probabilities of both types of hybrids were equal, the cytoplasmic background does not affect the male's behaviour and the dysfunctions in courtship behaviour are most likely due to disruption of nuclear (courtship) genes. Hybrids with *N. vitripennis* cytoplasm suffered less from spermatogenic sterility than those with *N. longicornis* cytoplasm, which is in line with hybrids of *N. vitripennis* and *N. giraulti* (Clark *et al.*, 2010). A role for cytoplasmic factors in inducing male (spermatogenic) sterility has been proposed previously (Ehrman, 1963; Mishra and Singh, 2005), but currently receives little scientific interest.

This is the first systematic study on sex-specific hybrid incompatibilities in a haplodiploid species pair. We found sex-specific hybrid incompatibilities, with heterozygous females suffering less than hemizygous males for hybrids of *N. vitripennis* and *N. longicornis*, which is largely consistent with limited data obtained from *N. vitripennis* and *N. giraulti* (Breeuwer and Werren, 1995). These results are in concordance with predictions by Haldane (1922) and Koevoets and Beukeboom (2009) about the presence of sex-specific hybrid incompatibilities in species with haplodiploid sex determination. We do, however, stress that the mechanisms that lead to Haldane's rule in haplodiploids can differ from those in diploids. As discussed below, cytonuclear incompatibilities seem to cause hybrid incompatibilities in *Nasonia*, and the disruption of the oxidative phosphorylation (OXPHOS) pathway is a likely candidate for causing hybrid mortality. If dominance has a large role in hybrid incompatibilities, then the cytonuclear interactions are unlikely to lead to hybrid incompatibilities in F_1 hybrids of diploid species. This follows from the fact that the nuclear OXPHOS genes are predominantly located on the autosomes (more than 84% of the nuclear OXPHOS genes are located on the autosomes in *Drosophila*; based on MitoDrome: http://mitodrome.ba.itb.cnr.it). Thus recessive incompatibilities between the autosomal genes and the cytoplasm would always be masked by heterozygosity in diploid F_1 hybrid offspring.

In this study we took the first step in studying Haldane's rule in *Nasonia*: We observed that diploid females suffer less from hybridization than haploid males. F_1 female hybrids are mostly viable and fertile, whereas a significant percentage of hybrid males are inviable and the few surviving hybrid males are mostly sterile. F_1 hybrid females and F_2 hybrid males of haplodiploid species are, however, not fully comparable in their genetic make-up. F_1 hybrid females inherit one intact genome complement from each parental species, that is, they are heterozygous for all diverged loci. F_2 hybrid males inherit a single recombined chromosome set from their mother and each locus has either an *N. vitripennis* or an *N. longicornis* allele. This difference in genetic make-up between males and females can be uniquely exploited for finding the underlying mechanisms of Haldane's rule in *Nasonia*. If only dominance effects are responsible for the hybrid incompatibilities, then heterozygous hybrid females are not expected to suffer from incompatibilities. Our results are consistent with these dominance effects; however, if faster-male effects solely explain the incompatibilities in this cross, then hybrid females are not expected to suffer from incompatibilities either. As F_1 diploid hybrid offspring possess one chromosome set from both parental species, the magnitude of hybrid incompatibilities in F_1 hybrids is highly dependent on dominance effects, regardless of the sex of the hybrid. Therefore, the next step in studying Haldane's rule in *Nasonia* and to distinguish between the dominance theory and the faster-male theory is to generate partly homozygous hybrid females by backcrossing F_1 hybrid females to both parental species. If dominance effects explain Haldane's rule in *Nasonia*, the obtained hybrid females should suffer from hybrid incompatibilities. However, if faster-male effects explain Haldane's rule instead, the obtained hybrid females should not suffer from hybrid incompatibilities. Note, however, that F_2 females derived from backcrosses will still be heterozygous for, on average, 50% of their genome. Therefore, the resulting mortality/sterility will be obscured by heterozygosity if dominance has a large role. A specific TRDL in F_2 haploid males will show a significant bias towards heterozygosity in F_2 backcross hybrid females under the dominance theory. By contrast, the faster-male theory does not predict a bias at such TRDL in females. Our preliminary data suggest that the TRDLs that we identified in this study have different effects in F_2 backcross females. This indicates that Haldane's rule is likely a composite phenomenon in *Nasonia*. A full analysis of these backcross F_2 females will be published later.

We have identified the genomic regions that are involved in causing F_2 hybrid male mortality by using microsatellite markers. Hybrid mortality in crosses between *N. vitripennis* and *N. longicornis* is highly genotype-specific. *N. vitripennis* nuclear genes on chromosomes 4 and 5 appear to be disrupted in their function if they are in *N. longicornis* cytoplasm and *N. longicornis* nuclear genes on chromosomes 1 and 5 are disrupted in their function if they are in *N. vitripennis* cytoplasm. This asymmetry of incompatibilities when studying reciprocal crosses is a common phenomenon for incompatibilities between few loci (Turelli and Moyle, 2007). Candidates for a stringent co-evolution of nuclear and cytoplasmic genes

are those coding for the OXPHOS pathway, whose efficacy is most likely also dependent on environmental factors (Rawson and Burton, 2002). Nuclear and mitochondrial genes are interacting in four out of five OXPHOS complexes (only complex-II consists of nuclear products only). Ellison *et al.* (2008) found that OXPHOS complexes in *Nasonia* hybrids are less functional than those of pure species. Gibson *et al.* (2010) mapped the nuclear-encoded OXPHOS genes in *Nasonia* and found that chromosomes 1, 2, 4 and 5 encode for OXPHOS genes with non-synonymous substitutions among *Nasonia* species. Whether these substitutions are responsible for the observed hybrid mortalities needs to be validated by functional analysis of the genes.

A study on hybrids of *N. vitripennis* and *N. giraulti* suggested nuclear–nuclear hybrid incompatibilities being partially responsible for hybrid mortality (Breeuwer and Werren, 1995). Here, we screened for nuclear–nuclear incompatibilities by means of analysing linkage disequilibrium between markers from different chromosomes. Our data revealed one significant deviation from the expected ratio of recombinant and non-recombinant genotypes between two markers, one on chromosome-2 and one on chromosome-4 in hybrids with *N. vitripennis* cytoplasm. As we found no linkage disequilibrium between these two markers in the reciprocal cross, the interdependency might be part of a higher order incompatibility that includes a cytoplasmically inherited genetic factor. Niehuis *et al.* (2008) also found only evidence for cytonuclear incompatibilities between *N. vitripennis* and *N. giraulti*. Our data and those of Niehuis *et al.* (2008) indicate that cytonuclear incompatibilities have a primary role in hybrid incompatibilities in *Nasonia*. Whether or not this is a unique characteristic of the *Nasonia* genus remains to be investigated. However, given that the mitochondrial genome typically evolves faster than the nuclear genome and that cytonuclear incompatibilities in *Nasonia* are likely recessive, it appears likely that cytonuclear incompatibilities are more widespread and may have only remained undetected in diploid organisms because of dominance effects (but see Burton *et al.*, 2006).

We have located TRDLs responsible for the hybrid incompatibilities between *N. vitripennis* and *N. longicornis*, which are in congruence with previous results from *N. vitripennis* and *N. giraulti*. Our data suggest nuclear loci on chromosomes 4 and 5 whose *N. vitripennis* allele is incompatible with an allospecific cytoplasm (that is, that of *N. longicornis*). Niehuis *et al.* (2008) postulated a similar locus on chromosome-4 only. Furthermore, we identified nuclear loci on chromosomes 1 and 5, which are incompatible with *N. vitripennis* cytoplasm when having an allospecific nuclear allele. Niehuis *et al.* (2008) again found similar results when studying *N. vitripennis* and *N. giraulti*. Although the currently available mapping data are not accurate enough to evaluate the evolution of hybrid incompatibilities in the *Nasonia* genus in detail, it appears that most of the mapped hybrid incompatibilities evolved prior to the split of *N. longicornis* and *N. giraulti*. This is supported by the lack of hybrid incompatibilities between these species (Bordenstein *et al.*, 2001). The true nature of the gene interactions can ultimately only be fully assessed by identifying the genes that underlie the hybrid incompatibilities.

We have found a large effect of the cytotype on F_2 hybrid male sterility and mortality, indicative of nuclear mitochondrial crosstalk (illustrated by our genetic analysis). Cytotype did not affect F_1 hybrid female sterility and mortality. The disruption of the nuclear–mitochondrial crosstalk could explain our finding of sex-specific hybrid incompatibilities in *Nasonia*. Different *Nasonia* species are infected by different strains of *Wolbachia* and these *Wolbachia* species seem to have caused mitochondrial sweeps during their spread (Raychoudhury *et al.*, 2010b). This could have strengthened the co-evolution of nuclear and mitochondrial genes in *Nasonia*. Furthermore, cytonuclear incompatibilities can have different effects on male and female mortality (for example, different energy requirements) and sterility (no role of mitochondria reported during oogenesis).

We have studied hybrid incompatibilities in F_1 and F_2 female and male hybrids of *N. vitripennis* and *N. longicornis*, and we have found that this genus of haplodiploid wasps follows a sex-specific pattern described by Haldane's rule. Hybrid incompatibilities in *Nasonia* cause both mortality and sterility, and the specific level is largely controlled by the cytoplasm, suggesting cytonuclear incompatibilities. Although haplodiploids have been ignored so far when studying Haldane's rule, our data suggest that haplodiploids at least follow the predictions of the rule in that the heterozygous sex suffers less than the hemizygous sex. We encourage other researchers to test these sex-specific hybrid incompatibilities in more haplodiploid systems in order to identify the generality of Haldane's rule and to facilitate the identification of the genetic mechanisms underlying Haldane's rule.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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